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Purification and Properties of Recombinant *Plasmodium falciparum* S-Adenosyl-L-Homocysteine Hydrolase[1]

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Recombinant S-adenosyl-L-homocysteine (SAH) hydrolase of the malaria parasite *Plasmodium falciparum* was expressed in *Escherichia coli*, purified to homogeneity and characterized. Comparison of the malaria parasite SAH hydrolase with that derived from the human gene indicated marked differences in k_{cat} values. The values of both forward and reverse reactions of *P. falciparum* SAH hydrolase are more than 21-fold smaller than those of the human enzyme. K_m values of the parasite and human SAH enzymes are 1.2 and 7.8 μM, respectively. On the other hand, IC_{50} values of neplanocin A, a strong inhibitor of SAH hydrolase and a growth inhibitor of *P. falciparum*, are 101 nM for the parasite enzyme and 47 nM for human enzyme. *P. falciparum* SAH hydrolase has been thought to be a target for a chemotherapeutic agent against malaria. This study may make it possible to develop a specific inhibitor for the parasite SAH hydrolase.

Key words: chemotherapeutic agent, malaria, neplanocin A, *Plasmodium*, S-adenosyl-homocysteine.

S-Adenosyl-L-homocysteine (SAH) hydrolase [EC 3.3.1.1] catalyzes the reversible hydrolysis of SAH to adenosine and L-homocysteine (*1*). SAH is produced by the biological methylation reaction performed by S-adenosylmethionine (SAM)-dependent methyltransferases and is degraded rapidly *in vivo* by SAH hydrolase. Since SAH strongly inhibits the methyltransferase, SAH hydrolase inhibition causes a down-regulation of the SAM-dependent methylation reaction as well as the accumulation of SAH (*2*). A number of SAH hydrolase inhibitors (*3*, *4*) have been designed for the purpose of preventing virus and tumor proliferation.

Neplanocin A (*5*), a strong inhibitor of SAH hydrolase, is reported to be a growth inhibitor of a malaria parasite, *Plasmodium falciparum*. Malaria is a leading cause of death worldwide and new chemotherapeutic agents are urgently needed because of the spread of drug-resistant strains of *P. falciparum*. Growth inhibition by neplanocin A has been observed in a drug-resistant strain (Camp/Malay) of *P. falciparum* as well as a drug-sensitive strain (FCR-3/Gambia) (*6*). Therefore, SAH hydrolase inhibitors are expected to provide new-type chemotherapeutic agents against malaria.

In order for antimalaria agents targeting SAH hydrolase to be developed, the parasitic enzyme should be well characterized. Creedon *et al.* (*7*) cloned a cDNA coding for *P. falciparum* SAH hydrolase and revealed that the parasite hydrolase contains a 41-amino acid insert (Gly145–Lys185) in its sequence. Since an analogous insert is not observed in mammalian SAH hydrolase, the enzymatic properties of human (*8*) and *P. falciparum* SAH hydrolases were expected to be different. However, the properties of the pathogenic parasite SAH hydrolase have not been characterized.

The present study describes the purification of recombinant *P. falciparum* SAH hydrolase, and its physicochemical and kinetic properties. As an aid to the development of chemotherapeutic agents against malaria, we also studied the inhibitory effects of neplanocin A on *P. falciparum* and human SAH hydrolase.

MATERIALS AND METHODS

Materials—*Escherichia coli* JM109, expression vector pKK223-3, Q-Sepharose FF, HiTrapQ, Blue-Sepharose, and Sephacryl S300 were from Amersham Pharmacia. GIGAPITE was from SEIKAGAKU Kogyo. S-Adenosyl-L-homocysteine was from Sigma. Neplanocin A was a gift from Asahi Chemical Industry (Osaka). Total *P. falciparum* (FCR-3) RNA was a generous gift from Professor Y. Wataya of Okayama University.

Constructions of the Expression Plasmids—A cDNA encoding the open reading frame of *P. falciparum* SAH hydrolase was amplified by reverse transcription-PCR from the total RNA of *P. falciparum* (FCR-3). Reverse transcription was carried out using SuperScriptII® reverse transcriptase as described in the user's manual (GIBCO). *Pfu* turbo DNA polymerase (Stratagene) and the following oligonucleotide primers (sense, 5′-ccggaattcatggttgaaaataag-3′ containing an *Eco*RI site; antisense 5′-ccgaagcttttaatatctgtattc-3′ containing a *Hin*dIII site) were used for PCR. To avoid false amplifications from genomic DNA contaminants in the total RNA preparation, reverse transcription-PCR was also carried out without the reverse transcription step.

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Abbreviations: SAH, S-adenosyl-L-homocysteine; SAM, S-adenosylmethionine.

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An open reading frame sequence of human SAH hydrolase was obtained in the same manner as described above except that total HepG2 cell RNA and the following oligonucleotide primers (sense, 5′-ccgaattccatgtctgacaaactgcc-3′ containing an *Eco*RI site; antisense 5′-ccaagctttcagtagcggtagtgatc-3′ containing a *Hin*dIII site) were used.

The PCR products digested with *Eco*RI and *Hin*dIII were ligated into the *Eco*RI and *Hin*dIII sites of the multiple cloning site of the pKK223-3 expression vector (Amersham Pharmacia). The insertion of the resulting vectors was subsequently verified by dideoxy sequencing. The resulting vectors containing *P. falciparum* SAH hydrolase cDNA and human SAH hydrolase cDNA were designated as pKKPfS and pKKHuS, respectively.

Bacterial Culture for the Production of SAH Hydrolase—*E. coli* (strain JM109) cells harboring pKKPfS were grown in 40 ml of 2 × YT medium containing 50 μg/ml ampicillin. After the cells reached late-logarithmic phase, a starter culture was inoculated into 2-liters of the same medium and incubated at 30°C. Isopropyl-β-D-galactopyranoside (0.5 mM) was added to the culture when the OD_{600} reached 0.4, and the bacteria were grown for 6 h at 30°C.

E. coli (strain JM109) cells harboring pKKHuS were grown in 5 ml of 2 × YT medium containing 50 μg/ml ampicillin. After the cells reached late-logarithmic phase, a starter culture was inoculated into 1-liter of the same medium and incubated at 37°C. Isopropyl-β-D-galactopyranoside (0.5 mM) was added to the culture when the OD_{600} reached 0.6, and the bacteria were grown for 10 h at 37°C.

Purification of P. falciparum SAH Hydrolase—All steps were carried out at 4°C.

Preparation of cell extracts (Step 1): The bacterial cells were collected from the 2-liter culture by centrifugation at 5,000 ×*g* for 15 min and stored at −20°C. The frozen cells were thawed and resuspended in 70 ml of buffer Ap [50 mM potassium phosphate buffer (pH 7.6), 5 mM EDTA, 2 mM 2-mercaptoethanol]. The suspension was subjected to sonication disruption (180 W output, 30 s × three times at 0°C). Cell debris was removed by centrifugation at 15,000 ×*g* for 15 min.

Ammonium sulfate precipitation (Step 2): Ammonium sulfate was added to the cell extract to 35% saturation (19.4 g/100 ml). The extract was then stirred for 1 h, and the precipitate was removed by centrifugation at 15,000 ×*g* for 15 min. The supernatant was adjusted to 60% saturation with ammonium sulfate (15.1 g/100 ml) to precipitate the enzyme. After stirring for 1 h, the precipitate was collected by centrifugation at 15,000 ×*g* for 15 min and dissolved in buffer Ap.

Gel filtration chromatography on Sephacryl S300 (Step 3): The solution from step 2 was applied to a column (90 × 2.6 cm) of Sephacryl S300 preequilibrated with buffer Ap at a flow rate of 30 ml/h. The protein was eluted with buffer Ap. Fractions containing the enzyme were pooled and used for further purification.

Q-Sepharose chromatography (Step 4): The fraction obtained in step 3 was applied to a column (16 × 2.5 cm) of Q-Sepharose FF preequilibrated in buffer Bp at a flow rate of 30 ml/h. The enzyme was not absorbed onto the resin. Fractions containing the enzyme were pooled and dialyzed against buffer Cp [20 mM TrisHCl (pH 7.5), 2-mM 2-mercaptoethanol].

Blue-Sepharose chromatography (Step 5): The fraction obtained in step 4 was applied to a column (10 × 2.5 cm) of Blue-Sepharose preequilibrated in buffer Cp at a flow rate of 30 ml/h. The enzyme was not absorbed onto the resin. Fractions containing the enzyme were pooled and used for further purification.

Hydroxyapatite chromatography (Step 6): The fraction obtained in Step 5 was applied to a GIGAPITE (1.2 × 8 cm) column preequilibrated in buffer Cp at a flow rate of 20 ml/h. Unbound protein was eluted with 100 ml of buffer Cp at a flow rate of 20 ml/h. The bound protein was eluted by a 100-ml linear gradient of 0–0.2 M potassium phosphate buffer (pH 7.5) in buffer Cp.

Storage: The fractions obtained from Step 6 were pooled and concentrated to 4 ml on a YM-10 membrane (Amicon) and stored at −20°C after the addition of glycerol to 20% (v/v) concentration.

Preparation of Human SAH Hydrolase—The method for the purification of human SAH hydrolase was a modification of earlier methods (*9*). All steps were carried out at 4°C.

Preparation of cell extract (Step 1): The bacterial cells were collected from the 1-liter culture by centrifugation at 5,000 ×*g* for 15 min and stored at −20°C. The frozen cells were thawed and resuspended in 70 ml of buffer Ah [50 mM potassium phosphate buffer (pH 7.2), 1 mM EDTA, 2 mM 2-mercaptoethanol]. The suspension was subjected to sonication (180 W output, 30 s × three times at 0°C). Cell debris was removed by centrifugation at 15,000 ×*g* for 15 min.

Ammonium sulfate precipitation (Step 2): Ammonium sulfate was added to the cell extract to 35% saturation (19.4 g/100 ml). After stirring for 1 h, the precipitate was removed by centrifugation at 15,000 ×*g* for 15 min. The supernatant was adjusted to 60% saturation with ammonium sulfate (15.1 g/100 ml) to precipitate the enzyme. After stirring for 1 h, the precipitate was collected by centrifugation at 15,000 ×*g* for 15 min and dissolved in buffer Ah.

Gel filtration chromatography on Sephacryl S300 (Step 3): The solution was applied to a column (90 × 2.6 cm) of Sephacryl S300 preequilibrated with buffer Ah at a flow rate of 30 ml/h. The protein was eluted with buffer Ah. Fractions containing the enzyme were pooled and diluted five-fold with 1 mM EDTA.

Q-Sepharose HP chromatography (Step 4): The diluted fraction in Step 3 was applied to a HiTrapQ (bed: 1 ml) column preequilibrated in buffer Bh at a flow rate 20 ml/h. The enzyme was not absorbed onto the resin. Fractions containing the enzyme were pooled.

Storage: The pooled fraction from Step 4 was concentrated to 5 ml on a YM-10 membrane (Amicon) and stored at −20°C after the addition of glycerol to 20% (v/v) concentration.

Assay of SAH Hydrolase—The hydrolysis of SAH was determined by measuring the formation of inosine using HPLC. Initial velocities of SAH hydrolysis were assayed at 30°C for 4 min in 0.1 ml of 25 mM potassium phosphate buffer (pH 7.2) containing 2.5–80 μM SAH, 0.4 unit of adenosine deaminase and SAH hydrolase. The reaction was started by the addition of 10 μl of SAH and terminated by the addition of 10 μl of 0.67 N HCl. The reaction mixture was kept on ice until HPLC analysis. The mixture was analyzed for inosine on a Shimadzu HPLC system equipped

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with a model SPD-6AV variable wavelength detector (detection wavelength was 260 nm) and CrestPak C18s column (150 × 4.6 mm, JASCO, Tokyo). The mobile phase was composed of 25 mM KH_2PO_4 and acetonitrile 95:5 (25 mM KH_2PO_4: acetonitrile). The flow rate was 0.8 ml/min. In the hydrolytic reaction, one unit of SAH hydrolase was defined as the amount resulting in the production of 1 μmol inosine/min at 30°C.

In the synthetic direction, the enzyme assay was a modification of an earlier method (*9*). The enzyme was incubated with 40 μM adenosine and 5 mM DL-homocysteine in 0.1 ml of 25 mM potassium phosphate buffer, pH 7.2, at 30°C for 2 min in the standard assay system. The reaction was started by the addition of 10 μl of SAH hydrolase and terminated by the addition of 10 μl of 0.67 N HCl. The reaction mixture was kept on ice until HPLC analysis. The mixture was analyzed for SAH on the Shimadzu HPLC system described above. In the synthetic reaction, one unit of SAH hydrolase was defined as the amount synthesizing 1 μmol of SAH/min at 30°C.

The peaks of inosine and SAH in the assay were identified by comparison with standard compounds.

Determination of E-NAD⁺ and E-NADH—The intrinsic cofactors of SAH hydrolase were analyzed by the method of Huang *et al.* (*10*). SAH hydrolase in 0.2 ml of 20 mM sodium phosphate buffer, pH 7.2, was added to 3 volumes of 97% ethanol to denature the enzyme. The precipitate was removed by centrifugation, and the supernatant was evaporated on a centrifugal concentrator. The residue was then dissolved in 0.1 ml of water. The sample (20 μl) was analyzed by the HPLC system described above. The mobile phase was composed of 0.1 M sodium phosphate, pH 7.0, and 5% methanol. The flow rate was 0.8 ml/min.

Other Procedures—Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 10% gels as described by Laemmli (*11*). Proteins in the gels were stained using a Silver Stain Kit II (Wako Pure Chemicals, Osaka). Protein concentration was determined using a protein assay reagent (Bio-Rad) with bovine serum albumin as the standard.

RESULTS

Expression and Purification—The SDS-PAGE analysis of the whole-cell proteins of *E. coli* harboring pKKPfS showed a specific protein band of 55 kDa that was not exhibited by *E. coli* harboring pKK223-3 (Fig. 1). The size is consistent with the calculated molecular mass of 53.9 kDa. Since the 55 kDa protein was in the insoluble fraction when expressed at 37°C, the cultivation temperature was lowered to 30°C. Lowering the temperature resulted in part of the enzyme being recovered in a soluble fraction. No *P. falciparum* SAH hydrolase activity, however, was detected in the cell extract, even though 3-deazaadenosine was used as a substrate according to the literature (*7*) (data not shown). SAH synthetic activity was detected only after the Sephacryl S300 step (Table I) because of the low expression efficiency of the recombinant enzyme. The 55 kDa protein was purified to homogeneity by a 6-step procedure as described in "MATERIALS AND METHODS" (Fig. 1). Approximately 1.4 mg of protein was obtained from a 2-liter culture (Table I). The purified protein was verified to be *P. falciparum* SAH hydrolase by the amino terminal sequence. No 3-deazaadenosylhomocysteine synthetic activity was detected using the purified and concentrated enzyme (data not shown).

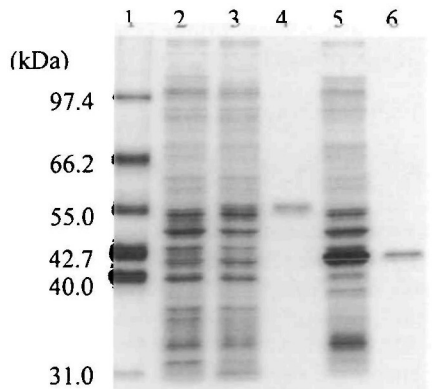

Fig. 1. **Expression of recombinant SAH hydrolase.** Lanes: 1, size markers; 2, whole-cell proteins from *E. coli* harboring pKK223-3; 3, whole-cell proteins from *E. coli* harboring pKKPfS; 4, purified *P. falciparum* SAH hydrolase; 5, whole-cell proteins from *E. coli* harboring pKKHuS; 6, purified human SAH hydrolase.

Human SAH hydrolase, 43 kDa migrating as a 43 kDa band on SDS-PAGE, was expressed in the soluble fraction at 37°C and purified as shown in Fig. 1. About 32 mg of homogeneous protein was obtained from a 1-liter culture (Table II).

Molecular Mass—The molecular masses of the purified *P. falciparum* and human SAH hydrolases under native conditions were established by gel filtration on a Superdex 200 column to be 200 and 150 kDa, respectively (data not shown). The results indicate that both *P. falciparum* and human SAH hydrolases consist of four identical subunits.

Effect of pH and Temperature on the Reaction Rate—In the SAH synthesis direction, the effect of pH was examined in 50 mM potassium phosphate buffer ranging from pH 5.0 to 8.0 (Fig. 2A). *P. falciparum* and human SAH hydrolases showed optimum activity around pH 7.6 and 7.4, respectively. Using 50 mM potassium phosphate buffer (pH 7.2), temperature optima were determined to be 55°C (*P. falciparum*) and 50°C (human) (Fig. 2B).

TABLE I. **Summary of the purification of *P. falciparum* SAH hydrolase.**

Step	Protein (mg)	Activity[a] (unit)	Specific activity (unit/mg)
Cell extract	245	n.d.	—
Ammonium sulfate precipitation	164	n.d.	—
Sephacryl S300	35	0.22	0.01
Q-Sepharose	5.7	0.39	0.07
Blue-Sepharose	2.0	0.21	0.10
Hydroxyapatite	1.4	0.16	0.11

[a]The activity was assayed in the synthetic direction.

TABLE II. **Summary of the purification of human SAH hydrolase.**

Step	Protein (mg)	Activity[a] (units)	Specific activity (unit/mg)
Cell extract	409	156	0.38
Ammonium sulfate precipitation	250	166	0.66
Sephacryl S300	117	126	1.1
Q-Sepharose	32	76.5	2.4

[a]The activity was assayed in the synthetic direction.

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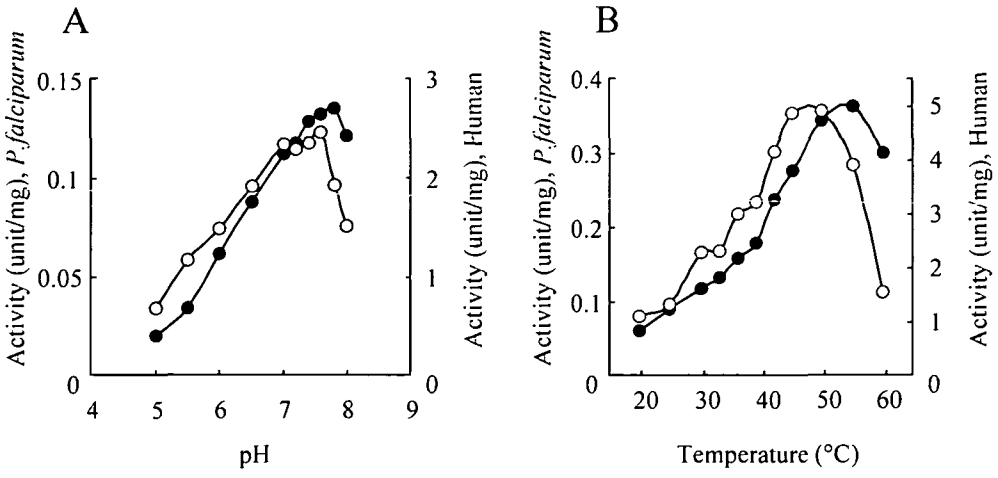

Fig. 2. **pH and temperature dependencies of SAH hydrolase activities.** SAH synthesis activities of purified *P. falciparum* SAH hydrolase (●) and human SAH hydrolase (○) were determined at the indicated pH (A) and temperature (B).

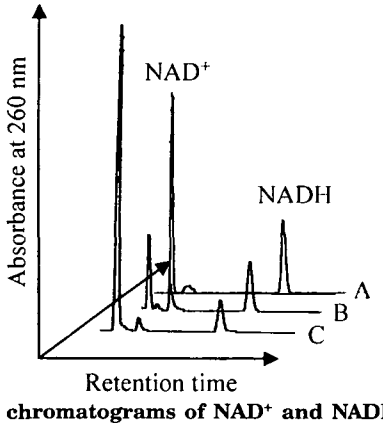

Fig. 3. **HPLC chromatograms of NAD⁺ and NADH.** NAD⁺ and NADH were released from the purified enzymes (20 μg each) and analyzed by HPLC as described under "MATERIALS AND METHODS." A, authentic NAD⁺ and NADH; B, *P. falciparum* SAH hydrolase; C, human SAH hydrolase.

TABLE III. **Comparative properties of *P. falciparum* and human recombinant SAH hydrolases.**

	P. falciparum	Human
Molecular mass (kDa)		
Native	200	150
Denatured	55	43
pH optimum	7.6	7.4
Temperature optimum (°C)	55	50
Coenzyme content (mol/mol subunit)		
NAD^+	0.27	0.65
NADH	0.47	0.19
Kinetic parameters[a]		
K_m (μM)		
SAH[b]	1.2 ± 0.1	7.8 ± 0.2
Adenosine[c]	0.6 ± 0.1	1.4 ± 0.2
DL-Homocysteine[d]	64.3 ± 4.7	111 ± 0.7
k_{cat} (min^{-1})		
Hydrolytic	1.4 ± 0.02	31.8 ± 0.5
Synthetic	5.2 ± 0.2	111 ± 3.7

[a]Values given are the averages from at least three experiments with standard errors. [b]Values were obtained from the reaction with an increased concentration of SAH (2.5–40 μM). [c]Values were obtained from the reaction with a fixed concentration of DL-homocysteine (5 mM) and an increased concentration of adenosine (1–8 μM). [d]Values were obtained from the reaction with a fixed concentration of adenosine (40 μM) and an increased concentration of DL-homocysteine (20–400 μM).

Cofactor Content of SAH Hydrolase—Intrinsic cofactors of SAH hydrolase were determined using the HPLC system (*10*). NAD^+ and NADH appeared at 6 min and 13 min, respectively, after the start of the injection (Fig. 3). *P. falciparum* SAH hydrolase contained 0.27 mol of NAD^+ and 0.47 mol of NADH per mol of subunit. On the other hand, human SAH hydrolase contained 0.65 mol of NAD^+ and 0.19 mol of NADH per mol of subunit.

Kinetic Properties of SAH Hydrolase—Michaelis-Menten-type kinetics was observed for both the hydrolytic and synthetic reactions of the enzymes. The kinetic parameters obtained from at least 3-times experiments are summarized in Table III. The k_{cat} values in both directions of the parasite enzyme were more than 20 times lower than the values for the human enzyme. The addition of NAD(H) to the standard assay system did not alter the values. All the K_m values were also lower in the parasite enzyme than in the human enzyme.

Inhibition by Neplanocin A—The inhibitory effect of neplanocin A on *P. falciparum* and human SAH hydrolase activity in the synthetic direction was measured. Neplanocin A inhibited the parasite enzyme in a dose-dependent manner, and the IC_{50} values for the *P. falciparum* and human enzymes under the conditions examined were 101 nM and 47 nM, respectively (Fig. 4).

DISCUSSION

In this study, we purified and characterized recombinant *P. falciparum* SAH hydrolase for the first time. Although the enzyme activity of the malaria parasite SAH hydrolase had been reported (*7*), some ambiguity lingered. First, a recombinant *E. coli* cell extract was used as the enzyme in the assay. Second, the reaction product was not assigned to the product (*S*-3-deazaadenosylhomocysteine), despite the assay being based on the formation of *S*-3-deazaadenosylhomocysteine from *S*-3-deazaadenosine and homocysteine. 3-Deazaadenosine is known to be a strong inhibitor of SAH hydrolase (*2*). On the other hand, our data clearly show that *P. falciparum* SAH hydrolase catalyses the hydrolysis and synthesis of the natural substrate, SAH.

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In the hydrolytic and synthetic reaction, the k_{cat} values of *P. falciparum* SAH hydrolase were more 20-fold lower than those of the human enzyme (Table III). The difference can be partly explained by the intrinsic NAD^+ content. SAH hydrolase catalyzes the hydrolysis or synthesis of SAH through oxidation and reduction cycles of the intrinsic NAD^+ (*12*); consequently the apo form and the NADH form of the enzyme show no enzymatic activity.

The NAD^+ content of *P. falciparum* SAH hydrolase was estimated to be 0.27 mol/mol subunit, in contrast to the 0.65 mol/mol subunit of the human enzyme. The difference in the NAD^+ content, 2.4-fold, is insufficient to explain the enzyme activity. Another factor might be the 41–amino acid insert. The SAH hydrolase from the parasite *Trichomonas vaginalis* also contains a similar insert sequence corresponding to the 41–amino acid insert in the *P. falciparum* enzyme. Purified recombinant SAH hydrolase from *T. vaginalis* is reported to have a specific activity of 0.32 unit/mg in the synthetic reaction at 37°C (*13*). This value is comparable to the value of the *P. falciparum* enzyme (0.16 unit/mg at 36°C) rather than the value of the human SAH hydrolase (3.0 units/mg at 36°C, Fig. 2). Therefore, the poor ratio of NAD^+ and the 41–amino acid insert might result in the low activity of *P. falciparum* SAH hydrolase.

On the other hand, no such remarkable difference was observed in the K_m values between the *P. falciparum* and human enzymes. *P. falciparum* SAH hydrolase shows about 51% sequence identity with the human enzyme (*7*) and forms a homotetrameric structure as does the human enzyme. Besides, the pH and temperature dependency profiles exhibit essentially the same pattern. Thus, the three-dimensional structure of the parasite enzyme is expected to be the same as that of the human enzyme. According to the three-dimensional structure of human SAH hydrolase (*14*), 14 amino acid residues (His55, Thr57, Glu59, Cys79, Ser83, Asp131, Glu156, Thr157, Asn181, Lys186, Asp190, Asn191, His301, and His353 in the numbering system of the human SAH hydrolase) are involved in the binding of an adenosine analogue (9-2′,3′-dihydroxycyclopentan-1′-yl)adenine. Since all 14 residues corresponding to the human enzyme are conserved in *P. falciparum* SAH hydrolase, the substrate binding would be performed in the same manner as observed in the human enzyme.

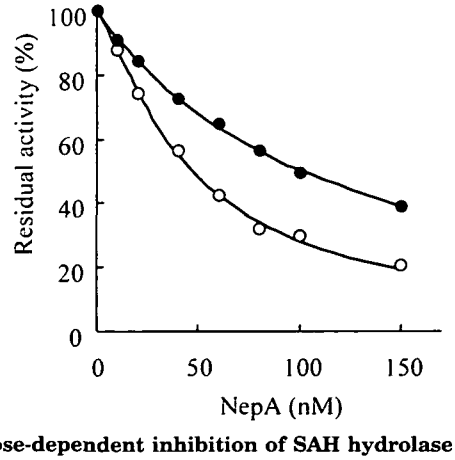

Fig. 4. **Dose-dependent inhibition of SAH hydrolase by neplanocin A.** Initial velocities for the synthesis of SAH were determined in the absence or the presence of neplanocin A. *P. falciparum* SAH hydrolase [0.05 unit/ml, 835 nM (●)] and human SAH hydrolase [0.06 unit/ml, 60 nM (○)] were used.

The inhibitory effect of neplanocin A on *P. falciparum* SAH hydrolase is also reported in this paper (Fig. 4). This suggests that growth inhibition of *P. falciparum* by neplanocin A is caused by the inhibition of the parasite SAH hydrolase. Therefore, targeting SAH hydrolase for the development of antimalaria agents is a rational strategy.

A number of SAH hydrolase inhibitors and their delivatives have been developed. Of these, some compounds might specifically inhibit *P. falciparum* SAH hydrolase to provide candidates for new-type chemotherapeutic agents against malaria. The recombinant *P. falciparum* SAH hydrolase reported here would facilitate the *in vitro* evaluation of inhibitor selectivity.

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